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Incompatibility Group P-1 *bla*$^+$ Plasmids Do Not Increase Penicillin Resistance of *Pseudomonas acidovorans*

D. L. BRUCE† AND R. A. J. WARREN*

Department of Microbiology, University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada

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Incompatibility group P-1 plasmids with the *bla*$^+$ genotype were transferred from various *Escherichia coli* strains to *Pseudomonas acidovorans* strain 29. When resistance to ampicillin was used as the criterion, none of these plasmids appeared able to express their Bla$^+$ phenotype in this host. When the plasmids were subsequently transferred back from these ampicillin-sensitive *P. acidovorans* transcipients to *E. coli* strains, it was found that the Bla$^+$ phenotype was again expressed. Although β-lactamase was not detected in cultures of *P. acidovorans* transcipients, macroiodometric determinations of β-lactamase activity made on broken cell suspensions revealed that β-lactamase was indeed synthesized. It was concluded that *P. acidovorans* strain 29 allows expression of the *bla* gene within the cell but that this organism is unable to excrete the enzyme.

Plasmids of the incompatibility (Inc) group P-1 have a very broad host range. RP1, for example, is transferrable to a wide variety of gram-negative bacteria (14). In general, the expression of resistance genes on these plasmids is host dependent. Although the level of resistance to a particular drug may vary, some degree of resistance is usually detectable in different hosts (1, 6). An exception was thought to occur with *Pseudomonas aeruginosa* 9169(R91) which seemed to transfer carbenicillin (Cb), kanamycin (Km), and tetracycline (Tc) resistance to *Escherichia coli* or *Shigella flexneri*, but to transfer only Cb resistance to other strains of *P. aeruginosa* (3). However, the *P. aeruginosa* 9169 strain in which R91 was isolated actually contains two plasmids: R91a, Inc P-1, determining resistance to Cb, Km, and Tc; and R91, Inc P-10, determining resistance to Cb only (10). R91 expresses its transfer functions well and is readily transferrable to *P. aeruginosa*; R91a, however, expresses its transfer functions poorly, and it is rarely transferrable to *P. aeruginosa* (10). Thus, it is not a host effect on plasmid phenotype, but rather the presence of two distinct plasmids which accounts for the anomalous behavior of *P. aeruginosa* 9169(R91) as a donor.

Recently, a derivative of RP1 was used to isolate amber suppressor mutants of *Pseudomonas acidovorans* strain 29 (21). Strain 29 is resistant to about 250 μg of ampicillin per ml, but RP1 does not raise this level of resistance. If anything, *P. acidovorans*(RP1) is slightly less resistant to ampicillin than the plasmid-free parent (21). RP1 is a very well-characterized plasmid, and it was transferred to *P. acidovorans* from an equally well-characterized *E. coli* strain. This made it unlikely that the donor strain carried two plasmids. Therefore, other Inc group P-1 plasmids with the Cb phenotype have been examined for their capacity to confer resistance to more than 250 μg of ampicillin per ml on *P. acidovorans* strain 29. The results presented in this paper show that the *P. acidovorans* plasmid-carrying strains have a *bla*$^+$ genotype and a Bla$^-$ phenotype. The Bla$^-$ phenotype results from an inability of *P. acidovorans* to excrete β-lactamase.

MATERIALS AND METHODS

Bacteria and plasmids. The strains and plasmids used are given in Tables 1 and 2.

Growth conditions and mating. The media used, the conditions of growth, and the mating conditions were described previously (21). The concentrations of drugs (micrograms per milliliter) used to select for transcipients were as follows: ampicillin, 1,000; kanamycin, 2,000; tetracycline, 35; and sulfanilamide, 250.

MICs. The minimal inhibitory concentration (MICs) of drugs were determined on solid medium as described previously (21).

β-Lactamase activity. Cultures were grown to a density of 5×10^8 cells per ml in broth. The cells from a sample of the culture were collected by centrifugation, washed once with phosphate-buffered saline and resuspended in the original growth volume of 40 mM phosphate buffer (pH 7.0) containing 1 mM dithiothreitol. The cells were ruptured by sonication at 4°C

† Present address: Department of Biology, University of Regina, Regina, Saskatchewan S4S OA2, Canada.

TABLE 1. *Bacterial strains*

Strain	Genotype	Source
E. coli		
JC3272	*trp his lys rpsL*	P. M. Bennett
JC2913	*thr leu*	J. L. Ingraham
J53	*pro met*	E. M. Lederberg
ECGC4682	prototroph Δ*lacZ*	B. J. Bachmann
P. acidovorans		
29	Prototroph	R. Y. Stanier

TABLE 2. *Plasmids*

Plasmid	Phenotype[a]	Reference
RP1	Km Am Tc	6
RK2	Km Am Tc	9
R26	Km Am Tc Sm Gm Su Cm Hg	19
R30	Am Km Tc Nm	3
R839	Km Am Tc Sm Su Hg	8
R906	Am Sm Su Hg	7

[a] Plasmid phenotypic characters indicate resistance to ampicillin (Am), kanamycin (Km), tetracycline (Tc), streptomycin (Sm), gentamycin (Gm), chloramphenicol (Cm), sulfonamide (Su), neomycin (Nm), and mercuric ion (Hg).

by using a Bronwill sonic oscillator. This crude extract was used directly in the standard iodometric assay (16, 18). The concentration of dithiothreitol in the buffer did not interfere with the assay. Another sample of the culture was assayed without any treatment.

Antibiotics. All antibiotics were purchased from Sigma Chemical Co., St. Louis.

RESULTS

Transfer of plasmids to *P. acidovorans*. Plasmids were transferred to *P. acidovorans* from various auxotrophic strains of *E. coli*. When selection was made for kanamycin-, tetracycline-, or sulfanilamide-resistant transcipients, normal, distinct colonies were obtained. With RP1, R26, R30, and RK2, selection for kanamycin resistance always yielded more transcipients than selection for tetracycline resistance (Table 3). However, all of the transcipients tested after selection on either of these drugs were resistant to both of them (Table 4).

When selection was made for ampicillin-resistant transcipients, dilutions of the mating mixtures which yielded distinct colonies on the other drugs gave films of growth on the ampicillin plates.

Properties of the transcipients. Transcipient clones were purified once by streaking out on the selective medium. Although colonies were not obtained on the ampicillin plates spread with the mating mixtures, the films of growth were touched at random with a fine wire for streaking on ampicillin plates.

When streaked out on the same medium, clones selected on kanamycin, tetracycline, and sulfanilamide gave colonies over the entire area of streaking. The ampicillin-grown samples tended to give heavy growth in the area of initial streaking, with very few single colonies at any distance from this heavy growth.

Single colonies were picked from the secondary plates with sterile applicator sticks and transferred to 5 ml of minimal medium without carbon source. Applicator sticks were used to spot from these suspensions onto plates of the selective and nonselective drugs, and onto drug-free minimal medium as a control.

All the transcipients selected on kanamycin and tetracycline were resistant to these drugs and apparently to ampicillin as well (Table 4). R906 transcipients selected on sulfanilamide were resistant to this drug and apparently to ampicillin (Table 4). However, cells purified from the ampicillin plates were mostly sensitive to the nonselective drugs (Table 4). Clones of plasmid-free *P. acidovorans* behaved like the transcipients selected on ampicillin.

The size of the inoculum transferred from the transcipient suspensions to the ampicillin plates was reduced by using a fine platinum wire instead of an applicator stick. Growth was obtained, but it took about 24 h longer than growth obtained on the plates that had been inoculated with applicator sticks. As observed previously (21), ampicillin resistance in *P. acidovorans* appeared to be inoculum dependent.

Most strains of gram-negative bacteria carrying Inc group P-1 plasmids are sensitive to phage PRD1 (15). All the transcipients selected on kanamycin, tetracycline, or sulfanilamide were

TABLE 3. *Frequency of transfer of plasmids to P. acidovorans*

E. coli donor	Frequency of transfer		
	Kanamycin (1,000 μg/ml)	Tetracycline (35 μg/ml)	Sulfanilamide (750 μg/ml)
JC3272(RP1)	1.4×10^{-4}[a]	3×10^{-5}	
JL2913(RK2)	2×10^{-3}	8×10^{-4}	
JC3272(R26)	7×10^{-6}	2×10^{-6}	
J53(R30)	2×10^{-5}	4×10^{-6}	
JC3272(R906)			4×10^{-4}

[a] Number of transcipients per recipient cell in the mating mixture at the time of plating. Dilutions of the mating mixtures yielding discrete clones on the other selective plates gave films of growth on plates with 1,000 μg of ampicillin per ml.

TABLE 4. *Properties of P. acidovorans strain 29 transcipients containing P-1 group plasmids*[a]

Plasmid	Transcipients selected with	Growth of transcipients on minimal medium containing the selection concn of drug				No. sensitive to phage PRD1	Transfer ampicillin resistance to *E. coli* (no.)
		Km	Am	Tc	Su		
RP1	Km	20	20	20		20	20
	Am	0	20	0		0	0
RK2	Tc	20	20	20		20	20
RK2	Km	20	20	20		20	20
	Am	5	20	5		5	5
	Tc	20	20	20		20	20
R26	Km	20	20	20		20	20
	Am	0	20	0		0	0
	Tc	20	20	20		20	20
R30	Km	20	20	20		20	20
	Am	0	20	0		0	0
	Tc	20	20	20		20	20
R839	Km	20	20	20		20	20
	Am	5	20	5		5	5
	Tc	20	20	20		20	20
R906	Su		20		20	20	20
	Am		20		0	0	0
	Sm		20		20	20	20

[a] A total of 20 transcipients from each cross were purified, and a single colony of each was transferred to 5 ml of minimal medium without carbon source. Sterile swab sticks were used to place drops of the suspensions on the test plates. Sensitivity to PRD1 was determined by cross-streaking the suspensions on the phage by using broth plates. The numbers of transcipients growing on the drug plates or sensitive to the phage or capable of transferring ampicillin resistance to *E. coli* are listed. See Table 2, footnote *a* for explanation of abbreviations.

sensitive to PRD1 (Table 4). Of the transcipients selected on ampicillin, only those also resistant to the nonselective drugs were sensitive to PRD1 (Table 4).

MIC of drugs. The ampicillin resistance of the *P. acidovorans* plasmid-carrying strains appeared to be inoculum dependent. Therefore, the MICs of drugs inhibiting colony formation by single cells were determined.

The various plasmids increased the resistance of *P. acidovorans* to all of the drugs tested with the exception of ampicillin (Table 5). In fact, the presence of the plasmids seemed to make *P. acidovorans* slightly more sensitive to ampicillin (Table 5).

Properties of Inc group P-1 plasmids after passage through *P. acidovorans*. The *P. acidovorans* transcipients described in Tables 4 and 5 were tested for their capacity to transfer ampicillin resistance back to *E. coli*. Selection for plasmid transfer was made on kanamycin or sulfanilamide, and a transcipient from each cross was then tested for ampicillin resistance. All the *P. acidovorans* transcipients selected on kanamycin, tetracycline, sulfanilamide, or streptomycin could transfer ampicillin resistance to *E. coli* (Table 4). However, as was expected, very few of the *P. acidovorans* transcipients selected on ampicillin could transfer ampicillin resistance to *E. coli* (Table 4). This confirmed that the *bla*$^+$ genotype of the Inc group P-1 plasmids tested is masked in some *P. acidovorans* strains.

To ensure that the plasmids were not altered by passage through *P. acidovorans*, some of them were transferred to *E. coli* 4682 from *P. acidovorans* and from the strains of *E. coli* used originally to transfer the plasmids to *P. acidovorans*. Then the MICs were determined for these *E. coli* 4682 plasmid-carrying strains. The results showed that the resistance profiles of the plasmids were unchanged by passage through *P. acidovorans* (Table 6).

Intracellular levels of β-lactamase in *P. acidovorans*. *P. acidovorans* plasmid-carrying strains could fail to express the plasmid-determined *bla*$^+$ genotype either by lack of expression of the *bla*$^+$ genes or by an inability to excrete β-lactamase into the periplasmic space. Accordingly, strain 29 and the RP1-carrying derivative were assayed for their content of the enzyme.

Neither strain 29 nor 29(RP1) excreted β-lactamase during growth in liquid medium (Table

TABLE 5. *MICs of antibiotics for P. acidovorans strain 29 with or without plasmids*

Plasmid	MIC[a]			
	Kanamycin	Ampicillin	Tetracycline	Sulfanilamide
None	100–250	200–300	0–5	500–1,000
RP1	2,000–3,000	100–200	50–100	500–1,000
RK2	2,000–3,000	100–200	50–100	500–1,000
R26	2,000–3,000	100–200	50–100	2,000–3,000
R30	2,000–3,000	100–200	50–100	500–1,000
R839	2,000–3,000	100–200	50–100	2,000–3,000
R906	100–250	100–200	0–5	2,000–3,000

[a] Concentrations, in micrograms per milliliter, between which single cells stopped forming colonies on both plates.

TABLE 6. *MICs for E. coli 4682 carrying plasmids from different donors*

Plasmid	Donor	MIC[a]		
		Kanamycin	Ampicillin	Tetracycline
None		<25	<100	<5
RP1	*E. coli* JC3272	50–100	>1,000	50–100
	P. acidovorans 29	50–100	>1,000	100–150
RK2	*E. coli* JC3272	50–100	>1,000	50–100
	P. acidovorans 29	50–100	>1,000	100–150
R26	*E. coli* JC3272	50–100	>1,000	100–150
	P. acidovorans 29	50–100	>1,000	100–150

[a] Concentrations, in micrograms per milliliter, between which single cells stopped forming colonies on broth plates.

7). However, cells of strain 29 contained the enzyme, and those of strain 29(RP1) contained several times the level of enzyme found in 29 (Table 7). *E. coli* 4682 was devoid of β-lactamase activity, but *E. coli* 4682(RP1) synthesized and excreted the enzyme (Table 7).

Effect of sucrose on the inoculum-dependent resistance of *P. acidovorans* to ampicillin. When large numbers of cells of *P. acidovorans* are spread on ampicillin-containing plates, many of the cells will die and release their β-lactamase. The enzyme will destroy the ampicillin in the vicinity of a lysed cell, thereby allowing the survival of any slowly growing cells. Sucrose stabilizes penicillin-induced spheroplasts. Overnight broth cultures of strain 29 and of various plasmid-carrying derivatives were serially diluted (10-fold dilutions) in broth, and 0.1-ml samples were spread on two sets of ampicillin-broth plates (1,000 μg of ampicillin/ml); one set of plates was unsupplemented, and the other contained 10% sucrose. In all cases, growth was inhibited at a 10-fold-higher cell concentration on the sucrose plates compared with that of the unsupplemented plates. Thus, the release of β-lactamase by lysing cells contributes to the inoculum-dependent resistance of *P. acidovorans* to ampicillin.

TABLE 7. *β-Lactamase activities[a] in various strains*

Strain	β-Lactamase activity[a]	
	Crude extract	Culture
E. coli 4682	<0.01	<0.01
E. coli 4682(RP1)	0.813	0.313
P. acidovorans 29	0.047	<0.01
P. acidovorans 29(RP1)	0.193	<0.01

[a] Expressed as micromoles of benzyl penicillin destroyed per minute per 5×10^8 cells. Cultures were grown in broth to 5×10^8 cells/ml.

DISCUSSION

It is clear that Inc group P-1 *bla*$^+$ plasmids are unable to express their *bla*$^+$ genotype in *P. acidovorans* 29. The *bla*$^+$ genes are not lost under these conditions, since the *bla*$^+$ genotype is readily transferrable from the plasmid-carrying *P. acidovorans* strain to an *E. coli* recipient.

The initial observation of the failure of an Inc group P-1 plasmid to enhance the ampicillin resistance of *P. acidovorans* was made during attempts to isolate amber suppressor mutants of *P. acidovorans*(pLM2) (21). pLM2 is a derivative of RP1 which contains amber mutations in

genes for ampicillin and tetracycline resistance (13). Mutants of *P. acidovorans*(pLM2) selected for the simultaneous development of resistance to ampicillin and tetracycline should carry amber suppressor mutations (13). However, none of the clones obtained on ampicillin-tetracycline plates after mutagenesis of *P. acidovorans*(pLM2) were amber suppressor strains (21). The reason for this is now obvious. The growth of any *P. acidovorans sup*(pLM2) mutants would not be inhibited by tetracycline, but they would be unable to excrete β-lactamase into the periplasm and would lyse in the presence of ampicillin. Furthermore, the *P. acidovorans sup*$^+$(pLM2) cells would not grow in the presence of tetracycline, so they would not lyse to release the chromosomally determined β-lactamase. Even though almost 50% of the clones selected on tetracycline alone contained amber suppressors (21) these cells would lyse on tetracycline-ampicillin plates.

The β-lactamase assays show that *E. coli* excretes the enzyme. The enzyme present in the culture will be in the periplasm of the cells and, to some extent, in the culture supernatant. *P. acidovorans* appears unable to excrete the β-lactamase(s) it synthesizes. This organism is resistant to about 250 μg of ampicillin per ml when it is plasmid free. This could result from the activity of the β-lactamase produced by this organism. However, some *Pseudomonas aeruginosa* strains are intrinsically resistant to some penicillins even though they produce little or no β-lactamase. This resistance seems to result from poor penetration of the outer membrane by the penicillins (17). Although this may also be true of *P. acidovorans*, its resistance to penicillins should be raised by an increased level of β-lactamase in the periplasm since RP1 increases the penicillin resistance of *P. aeruginosa* (6, 13, 14). RP1 does increase the amount of enzyme in *P. acidovorans* without increasing its resistance, and this suggests very strongly that the enzyme is not excreted. In some strains of *E. coli*, penicillins can penetrate the outer membrane so rapidly that β-lactamase, even though produced by the cell, may be incapable of inactivating the antibiotic with sufficient speed to prevent inhibition of cell wall biosynthesis (5). However, such intact cells should still exhibit β-lactamase activity.

The β-lactamase activity in *P. acidovorans* (RP1) is considerably less than that in *E. coli* 4682(RP1). The β-lactamase encoded by plasmid R6K appears to be synthesized with a sequence of 23 mostly hydrophobic amino acids at the N-terminal end which do not appear in the mature, secreted protein (20). The signal hypothesis (2) suggests that the excretion of proteins through membranes is facilitated by the presence of a hydrophobic amino terminus on nascent polypeptides. This leader sequence is cleaved from the primary translation product, or proenzyme, during or after excretion, to yield the active enzyme. The leader sequence does not necessarily reduce the enzymatic activity of a polypeptide: the pro-β-lactamase of *Bacillus licheniformis* has full enzymatic activity (4). Nonetheless, it is possible that *P. acidovorans* accumulates a pro-β-lactamase with reduced enzymatic activity. Alternatively, *P. acidovorans* may allow only poor expression of the plasmid-encoded β-lactamase genes and accumulate the active enzyme, not the proenzyme. In either case, the cell appears incapable of excreting the enzyme.

If this masking of the *bla*$^+$-transmissible plasmid-borne genotype is at all common in gram-negative bacteria, it means that some apparently penicillin-sensitive bacteria could be a reservoir of penicillin resistance for others.

The plasmids used determine several types of β-lactamase: TEM-1 by R839; TEM-2 by RP1; and OXA-2 by R906. The TEM-1 and TEM-2 type enzymes are quite similar; the OXA-2 enzymes seem quite different (11, 12). It is interesting that *P. acidovorans* seems unable to excrete neither of these plasmid-coded enzymes, nor its own (chromosomally determined?) enzyme. We are in the process of characterizing the *P. acidovorans* β-lactamase and the plasmid-determined enzymes accumulated by the various plasmid-carrying strains as a preliminary to examining the factor(s) preventing their excretion.

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